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Biosynthesis of long-chain polyunsaturated fatty acids in the razor clam *Sinonovacula constricta*: characterization of four fatty acyl elongases and a novel desaturase capacity

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ABSTRACT

As an unusual economically important aquaculture species, *Sinonovacula constricta* possesses high levels of long-chain polyunsaturated fatty acids (LC-PUFA). Previously, our group identified fatty acyl desaturases (Fad) with Δ5 and Δ6 activities in *S. constricta*, which was the first report of Δ6 Fad in a marine mollusc. Here, we further successfully characterize elongases of very long-chain fatty acids (Elovl) in this important bivalve species, including one Elovl2/5, two Elovl4 isoforms (a and b) and a novel Elovl (c) with Elovl4 activity. In addition, we also determined the desaturation activity of *S. constricta* Δ6 Fad toward 24:5n-3 to give 24:6n-3, a key intermediate in docosahexaenoic acid (DHA) biosynthesis. Therefore, *S. constricta* is the first marine mollusc reported to possess all Fad and Elovl activities required for LC-PUFA biosynthesis via the 'Sprecher pathway'. This finding greatly increases our understanding of LC-PUFA biosynthesis in marine molluscs. Phylogenetic analysis by interrogating six marine molluscan genomes, and previously functionally characterized Elovl and Fad from marine molluscs, suggested that DHA biosynthetic ability was limited to a few species, due to the general lack of Δ4 or Δ6 Fad in most molluscs.

Keywords: docosahexaenoic acid biosynthesis; fatty acyl desaturases; elongation of very long-chain fatty acids protein; Long-chain polyunsaturated fatty acids; *Sinonovacula constricta*

1. Introduction

In vertebrates, long-chain (≥ C_{20}) polyunsaturated fatty acids (LC-PUFA), especially arachidonic acid (20:4n-6, ARA), eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA), are key components of cellular membranes, determining their properties, and also serve as precursors of eicosanoids, affecting signal transductions [1, 2]. Moreover, LC-PUFA and their metabolites are essential for various physiological processes including neurological development and the immune response, and can be beneficial in mitigating several pathologies [3-5]. Provision of LC-PUFA in vertebrates can be via the diet directly or through endogenous production (biosynthesis) from dietary essential C_{18} polyunsaturated fatty acids (PUFA), linoleic acid (18:2n-6, LA) and *α*-linolenic acid (18:3n-3, ALA) [6]. Two types of enzymes, namely elongation of very long-chain fatty acid (Elovl) proteins and front-end desaturases play major roles in vertebrate LC-PUFA biosynthetic pathways [6]. Elovl enzymes are rate-limiting enzymes in fatty acid (FA) elongation pathways and catalyze the condensation reaction [6, 7]. Of the seven members of the Elovl family (Elovl1-7) described in vertebrates, only Elovl2, Elovl4 and Elovl5 have demonstrated elongation ability towards PUFA substrates [6, 7]. Typically, Elovl5 exhibits high activity toward $C_{16\text{-}20}$ PUFA substrates, Elovl2 shows high activity toward $C_{20\text{-}22}$ PUFA, whereas Elovl4 is involved in the biosynthesis of very long-chain PUFA (VLC-PUFA; > C_{24}) present especially in retina [8, 9]. In addition to elongases, vertebrates possess two distinct front-end desaturases termed Fads1 and Fads2 primarily with Δ5 and Δ6 desaturase activity, respectively [6]. Typically, Fads1 plays a key role in the

biosynthesis of ARA and EPA by desaturation of 20:3n-6 and 20:4n-3, respectively. Fads2 is involved in the desaturation of LA and ALA to give 18:3n-6 and 18:4n-3, respectively. Importantly, Fads2 also catalyze the rate-limiting step in DHA biosynthesis by conversion of 24:5n-3 to produce 24:6n-3, which is then partially *β*-oxidized to DHA via the Sprecher pathway [10].

Unlike vertebrates, the pathways of LC-PUFA biosynthesis in invertebrates have been less investigated [11, 12]. However, marine molluscs are arguably the group of aquatic invertebrates in which the biosynthesis of LC-PUFA has been most extensively investigated [11-13], partly driven by their critical roles in aquatic ecology and trophic cascade, as well as their importance as sources of health-promoting LC-PUFA for human consumers [14]. Marine molluscs have been demonstrated to possess genes encoding enzymes with roles in the biosynthesis of LC-PUFA [11-13, 15]. Interestingly, the biosynthetic capability of LC-PUFA in marine molluscs varies greatly among species and depends highly on their complement of desaturase and elongase genes, as well as their enzymatic activities [12]. Two different types of Elovl with roles in LC-PUFA biosynthesis have been characterized from marine molluscs. More specifically, an enzyme called “Elovl2/5”, regarded as an ancestral protein of the vertebrate Elovl2 and Elovl5 [16], has been characterized from the cephalopods *Octopus vulgaris* and *Sepia officinalis* [17, 18], and the bivalves *Chlamys nobilis* and *Crassostrea angulate* [19, 20]. Furthermore, Elovl4 orthologs have been characterized from *C. nobilis* and *O. vulgaris* [21, 22]. Meanwhile, two different fatty acyl desaturases (Fad) involved in LC-PUFA biosynthesis have been identified from marine molluscs, including Δ5 Fad from

O. vulgaris [23], *S. officinalis* [18], *C. nobilis* [24] and the gastropod *Haliotis discus hannai* [25], as well as a Δ8 Fad from *C. nobilis* [21].

As an economically important bivalve species, the razor clam *Sinonovacula constricta*, is widely distributed in estuarine and intertidal zones along the coasts of the West Pacific Ocean. It is one of the five principal marine molluscs in the global aquaculture industry, with a total production of over 823,000 tons, with a value of US$ 1.3 billion in 2016 [26]. From a nutritional point of view, *S. constricta* is a good source of health-promoting omega-3 (or n-3) LC-PUFA, especially DHA, which accounts for about 10% of the total FA [27]. Recently, three Fad were successfully characterized from *S. constricta* [28], two of which showed Δ5 desaturase activity and will be referred to as "Δ5 Fad_a" and "Δ5 Fad_b". A third Fad enzyme from *S. constricta* had Δ6 desaturase activity and will be termed herein as "Δ6 Fad". Importantly, *S. constricta* Δ6 Fad, the first report of a Δ6 desaturase in a marine mollusc, was able to desaturate both 18:3n-3 and 18:2n-6, but activity towards 24:5n-3, necessary for the Sprecher pathway [10], was not tested [28]. Moreover, no functional characterization of Elovl was reported in *S. constricta*.

The present study aimed to systematically characterize the complete repertoire of Elovl with putative roles in LC-PUFA biosynthesis pathway in *S. constricta*. Here we report on the molecular and functional characterization of four distinct Elovl genes, including one Elovl2/5, two Elovl4 (a and b isoforms) and a novel Elovl (termed Elovl_c). Furthermore, we investigated the ability of *S. constricta* Δ6 Fad to desaturate 24:5n-3 to 24:6n-3, so that we could establish whether this species has the potential to

operate the Sprecher pathway.

2. Materials and methods

2.1. Full-length cloning of S. constricta Elovl cDNAs

Total RNA was extracted from mixed tissues including muscle, gill and gonad of adult *S. constricta* specimens (55.23 ± 3.31 mm × 17.82 ± 1.21 mm, shell length × shell width; mean ± SD, n = 6) using MiniBEST Universal RNA Extraction Kit (TaKaRa, Japan). RNA quality was determined by running a subsample (~600 ng) on a 1 % agarose gel and RNA concentration was measured using a NanoDrop® ND-1000 (NanoDrop, USA). One μg of total RNA was reverse transcribed to cDNA using PrimeScript™ RT-PCR Kit (TaKaRa) and the resulting cDNA was used as template to isolate the full-length cDNA sequences of *S. constricta* Elovl by polymerase chain reaction (PCR).

First fragment *S. constricta* cDNA sequences with high homology to Elovl2/5 and Elovl4 sequences of vertebrates or marine molluscs, including one Elovl2/5, one pair of Elovl4 isoforms (a and b) and a novel Elovl (c), were obtained by searching against the transcriptome data. Moreover, gene specific primers (series of primer pairs of V-F and V-R, Table 1) were designed using Primer 5 to verify the target fragments. PCR was conducted using LA Taq® Hot Star Version (TaKaRa) (Table S1) and purified PCR fragments (Cat#DP1722, BioTeke, Co., Ltd, Beijing, China) were sequenced (BGI Tech Co., Ltd, Shanghai, China). Sequences of putative Elovl cDNAs were used to design gene-specific primers for rapid amplification of cDNA ends (RACE)-PCR

(SMARTer® RACE 5´/3´ Kit, Clontech, USA) to obtain full-length cDNAs (Table 1). The RACE-PCR analyses were performed with two-round nested PCR using a combination of gene-specific primers and primers designed on the RACE adaptor (Clontech) (Table S1). Potentially positive RACE-PCR fragments were sequenced as above. Finally, full-length cDNAs of four *S. constricta* Elovl cDNAs were obtained by aligning the first fragment with 5´ and 3´ RACE-PCR fragments (ClustalW 2.1) [29]. Where necessary purified PCR products were cloned into pMD™ 18-T Vector (TaKaRa) and transformed into *E. coli* DH5*α* competent cells. The recombinant single colonies successfully grown in LB plates containing ampicillin were selected, incubated and sequenced as above.

2.2. Retrieval of candidate Elovl and Fad sequences from six marine molluscan genomes

To examine the distribution and evolutionary diversity of marine mollusc genes encoding Elovl and Fad with potential roles in LC-PUFA biosynthesis, an extensive search was performed against currently available marine molluscan genomes from *Aplysia californica* (NCBI, genome ID: 443), *Crassostrea gigas* (NCBI, genome ID: 10758), *Crassostrea virginica* (NCBI, genome ID: 398), *Lottia gigantea* (NCBI, genome ID: 15113), *Mizuhopecten yessoensis* (NCBI, genome ID: 12193) and *Octopus bimaculoides* (NCBI, genome ID: 41501). Briefly, we first retrieved protein sequences from all the putative Elovl and Fad from corresponding genomes based on functional annotations. Subsequently, identical sequences of the same gene were discarded, and

the longest sequence was reserved for subsequent analysis. Finally, all hits of the putative Elovl sequences were selected by the presence of the diagnostic histidine box (H**HH) conserved in all members of the Elovl family [30]. Furthermore, selection of putative Fad sequences was based on the presence of the three histidine boxes (H***H, H**HH and QIEHH) and N-terminal cytochrome b5 domain with the heme-binding motif HPGG conserved in front-end desaturases [30]. The retrieved Elovl and Fad sequences from the genomes of the six molluscan species are shown in Table S2.

2.3. Sequence and phylogenetic analyses of S. constricta Elovl sequences

The deduced amino acid (aa) sequences of the newly cloned *S. constricta* Elovl2/5, Elovl4_a, Elovl4_b and Elovl_c were aligned by using ClustalW 2.1 [29]. A phylogenetic tree was constructed (MEGA 7) [31] with the deduced aa sequences from the newly cloned *S. constricta* Elovl cDNA sequences, and Elovl protein sequences of vertebrates and marine molluscs both retrieved *in silico* (Table S2) or functionally characterized in previous studies (Table S3). The phylogenetic analysis was performed with the maximum-likelihood approach, with confidence in the resulting phylogenetic tree branch topology measured by bootstrapping through 1,000 iterations. At the same time, a phylogenetic tree regarding marine molluscan Fad was constructed using the same method.

2.4. Functional characterization of S. constricta Elovl by heterologous expression in yeast

PCR fragments corresponding to the open reading frames (ORF) of the *S. constricta* Elovl sequences were amplified by PrimeScript™ Π High Fidelity RT-PCR Kit (TaKaRa) (Table S1) using primers containing restriction sites for *Kpn*I and *Eco*RI (underlined in Table 1). The resulting PCR fragments were purified, digested with the corresponding restriction endonucleases (New England BioLabs, USA) and inserted into similarly digested pYES2 vector (Invitrogen, USA) using DNA Ligation Kit (TaKaRa). Subsequently, the resulting recombinant plasmids pY*elovl2/5*, pY*elovl4_a*, pY*elovl4_b* and pY*elovl_c* were first transformed into *E. coil* DH5*α* competent cells to produce plasmid preparations for each Elovl gene. Plasmid preparations with correct sequences were subsequently transformed into yeast *Saccharomyces cerevisiae* InvSc1 competent cells using the S.c. EasyComp Transformation Kit (Invitrogen, USA). A control treatment consisting of yeast transformed with the empty pYES2 vector was also run. Selection of transformant yeast was performed on *S. cerevisiae* minimal medium$^{-uracil}$ (SCMM$^{-uracil}$) plates. One single colony of yeast transformed with either pY*elovl2/5*, pY*elovl4_a*, pY*elovl4_b*, pY*elovl_c* or empty pYES2 vector were used in each experiment. Yeast were first cultured in SCMM$^{-uracil}$ broth for 24 h. Subsequently, the cultures were centrifuged at 500 g for 2 min at room temperature. The precipitated yeast was further diluted with induction medium consisting of SCMM$^{-uracil}$ broth and 2 % galactose to reach OD600 = 0.4. At this point, one of the potential PUFA substrates, 18:2n-6, 18:3n-3, 18:3n-6, 18:4n-3, 20:4n-6, 20:5n-3, 22:4n-6 or 22:5n-3, were added. The FA substrate concentration was established at 0.5 mM (C_{18}), 0.75 mM (C_{20}) and 1 mM (C_{22}) as uptake efficiency decreases with increasing chain [32]. Finally, after

incubation at 30 °C for 48 h, yeast was harvested by centrifugation at 1,000 g for 1 min, washed twice with 5 mL ice-cold Hanks's Balanced Salt Solution (Invitrogen, USA) and freeze-dried for further FA analysis.

2.5. Determination of Δ6 desaturase activity of the S. constricta Δ6 Fad towards 24:5n-3 in co-transformant yeast

Previously, a Fad from *S. constricta* was shown to desaturate C_{18} PUFA at the Δ6 position [28]. However, it remained unclear whether this enzyme could also act as a Δ6 desaturase towards 24:5n-3 and thus contribute to DHA biosynthesis through the Sprecher pathway [10]. Following the same procedure as described by Oboh et al [33], ScInv1 *S. cerevisiae* were transformed with the constitutive expression plasmid p415TEF containing the ORF of *Danio rerio* Elovl2 (NP_001035452) and the inducible expression plasmid pYES2 containing the ORF of *S. constricta* Δ6 Fad (MH220406). The yeast assay was run as detailed by Oboh et al [33], with 22:5n-3 supplied at 1 mM concentration. A control treatment of co-transformed recombinant yeast grown in the presence of 18:3n-3 was also run. Yeast samples were collected and processed as described above for elongase assays.

2.6. FA analyses of yeast

Total lipids extracted from freeze-dried yeast samples [34] collected from the *S. constricta* Elovl2/5 and Δ6 Fad assays were used to prepare fatty acid methyl esters (FAME) as described by Xu et al [35], and FAME identified as described by Ran et al

[28]. FAME of samples from functional characterization assays of *S. constricta* Elovl4_a, Elovl4_b and Elovl_c were prepared as described by Oboh et al [36]. Identification of FAME from PUFA up to C_{24} was carried out by comparing retention times of peaks from known standards and databases (NIST 14.L). For VLC-PUFA, the response values of FAME were obtained by using the *m/z* ratios 79.1, 108.1 and 150.1 in the single ion monitoring (SIM) mode operated GC-MS [37]. All organic solvent mixtures used during lipid extraction and FAME preparation processes contained 0.01% (w/v) butylated hydroxytoluene (BHT, Sigma, China) to prevent oxidation. For elongase assays, conversion of PUFA substrates was calculated by the step-wise proportion of FA substrate converted to elongated product as [areas of first product and longer chain products / (areas of all products with longer chain than substrate + substrate area)] × 100 %. For *S. constricta* Δ6 Fad, the ability to convert 24:5n-3 to 24:6n-3 was calculated as [area of 24:6n-3 / (area of 24:6n-3 + area of 24:5n-3)] × 100 %. A similar equation was used to calculate the conversion of 18:3n-3 to 18:4n-3 established as a positive control in the *S. constricta* Δ6 Fad assay [33].

2.7. Tissue distribution of S. constricta Elovl mRNA

Tissue distribution of the newly cloned *S. constricta* Elovl mRNA was analyzed by quantitative real-time PCR (qPCR). Adult specimens (n = 18) of *S. constricta* (55.23 ± 3.31 mm × 17.82 ± 1.21 mm, shell length × shell width, mean ± SD) were purchased from a local market in Ningbo, China. After acclimation for 3 days to allow evacuation of the intestinal tract, total RNA was extracted from 10 tissues including mantle, labial

palps, inhalant siphon, exhalant siphon, foot muscle, intestine, gill, digestive glands, gonad and heart. Each tissue was sampled from six individuals and pooled, and triplicate samples prepared. One μg of total RNA was reverse transcribed to cDNA using PrimeScript™ RT Master Mix (Perfect Real Time, TaKaRa). The qPCR was conducted on a quantitative thermal cycler (Mastercycler ep realplex, Eppendorf, Germany) using SYBR® *Premix Ex Taq*™ Ⅱ (Tli RNaseH Plus) (TaKaRa) (Table S1) and primers as shown in Table 1. Both *β*-actin and 18S rRNA were selected as reference genes (Table 1). The relative expression of *S. constricta* Elovl was calculated by the $2^{-\Delta\Delta CT}$ method [38], and presented as the geometric means of qRT-PCR results derived from the expression of the two reference genes. Expression of candidate Elovl genes in all tissues were calculated in relation to that of foot muscle, which showed the lowest expression level among all tissues considered.

2.8. Statistical Analysis

Relative tissue distribution of *S. constricta* Elovl mRNA was analyzed by one-way analysis of variance (ANOVA), followed by Newman-Keuls tests (IBM SPSS Statistics 22.0 software, USA). A P value < 0.05 was considered statistically significant. Gene expression data were represented as means and standard deviation (n = 6).

3. Results

3.1. Sequences and phylogenetics of the S. constricta Elovl

The ORF of *S. constricta* Elovl2/5 was 933 bp encoding a polypeptide of 310 aa

(Fig. 1). The ORFs of *S. constricta* Elovl4_a and Elovl4_b were both 876 bp encoding polypeptides of 291 aa, whereas the ORF of *S. constricta* Elovl_c contained 912 bp encoding 303 aa (Fig. 1). Notably, *S. constricta* Elovl4_a and Elovl4_b shared high identity (89.69 %) in terms of aa sequence, with the main different aa region between them being highlighted with a bold line square in Fig. 1. The detailed sequences of *S. constricta* Elovl2/5, Elovl4_a, Elovl4_b and Elovl_c were deposited in the GenBank database with accession numbers MK134691-MK134694, respectively.

The phylogenetic results showed that the *S. constricta* Elovl2/5 was grouped together with functionally characterized Elovl2/5 from *O. vulgaris*, *S. officinalis* and *C. nobilis*, as well as Elovl2-like and Elovl5-like from the six examined marine molluscs (Fig. 2). Together, they formed a cluster close to vertebrate Elovl2 and Elovl5. Similarly, *S. constricta* Elovl4_a and Elovl4_b were grouped together with functionally characterized molluscan Elovl4 from *C. nobilis* and *O. vulgaris*, as well as Elovl4-like from the six examined marine molluscs (Fig. 2). Together, they formed a cluster close to vertebrate Elovl4. In contrast, *S. constricta* Elovl_c and some Elovl-like from five examined marine molluscs other than *L. gigantea* were grouped together (Fig. 2), which were most closely related to vertebrate Elovl1 and Elovl7.

3.2. Functional characterization of newly cloned S. constricta Elovl cDNAs

The FA composition of yeast transformed with empty pYES2 vector contained only the yeast endogenous FA (mainly 16:0, 16:1n-7, 18:0, 18:1n-9 and 26:0), together with the corresponding exogenously added PUFA. In contrast, additional PUFA were

identified in yeast transformed with all four Elovl sequences cloned from *S. constricta*.

When yeast was transformed with pY*elovl2/5* (Table 2, Fig. S1), elongation activity toward C_{18} and C_{20} PUFA substrates was observed, indicating that *S. constricta* Elovl2/5 possesses typical vertebrate Elovl5 activity and exhibited higher affinity toward n-6 PUFA compared to n-3 PUFA. However, *S. constricta* Elovl2/5 showed no elongation activity towards C_{22} PUFA substrates (i.e., 22:4n-6 and 22:5n-3). Furthermore, *S. constricta* Elovl2/5 also displayed some elongation activity towards monoenes, since the yeast endogenous FAs 16:1n-7 and 18:1n-9 were elongated to 18:1n-7 and 20:1n-7, and 20:1n-9 and 22:1n-9, respectively (data not shown).

When yeast were transformed with pY*elovl4_a*, pY*elovl4_b* and pY*elovl_c* (Table 3, Fig. S2), all the exogenously added PUFA substrates were elongated to different extents. On one hand, low levels of C_{18} PUFA were elongated to longer products up to C_{22} in the case of Elovl_c (Table 3). On the other hand, *S. constricta* Elovl4_a, Elovl4_b and Elovl_c exhibited higher conversions towards C_{20} and C_{22} PUFA substrates, which were elongated in some instances up to products with C_{34} (Table 3). Importantly, the three elongases were all able to elongate 22:5n-3 to 24:5n-3, a key intermediate in DHA biosynthesis via the Sprecher pathway.

3.3. Determination of Δ6 activity of S. constricta Δ6 Fad towards 24:5n-3 in co-transformant yeast

The control treatment consisting of yeast co-expressing the *D. rerio* Elovl2 and the *S. constricta* Δ6 Fad showed that the exogenously added substrate (18:3n-3) was

elongated to 20:3n-3 by the action of *D. rerio* Elovl2 and, importantly, was also desaturated to 18:4n-3 confirming the previously reported Δ6 desaturase activity (Fig. 3A) [28]. When co-transformant yeast were incubated in the presence of 22:5n-3, a peak corresponding to the elongation product 24:5n-3 was detected and a further peak identified as 24:6n-3 was also detected (Fig. 3B), denoting a Δ6 desaturase product from 24:5n-3.

3.4. Tissue distribution of S. constricta Elovl mRNA

Transcripts of *S. constricta* Elovl encoding genes were detected in all examined tissues (Fig. 4). Specifically, the highest expression of *S. constricta* Elovl2/5 was detected in digestive gland, followed by intestine and labial palps, with relatively low expression in other tissues. *S. constricta* Elovl4_a was mostly expressed in gill, followed by labial palps, and gonad, whereas Elovl4_b was highly expressed in labial palps and gill, followed by intestine. The highest expression of *S. constricta* Elovl_c was detected in mantle, followed by gill, labial palps and intestine.

4. Discussion

The deduced aa sequences of *S. constricta* Elovl all contained a diagnostic histidine box, which is conserved in all Elovl family members [30], indicating that Elovls have functional regions highly conserved during evolution. Notably, all four sequences contained a diagnostic “Q” (glutamine) in position -5 from the H**HH, characteristic of PUFA elongases and which is not present in non-PUFA elongases [30], indicating

that they all may play possible roles in PUFA elongation. Consistent with this, the elongases were demonstrated to elongate PUFA substrates by heterologous functional characterization. Interestingly, two Elovl4 isoforms (Elovl4_a and Elovl4_b) were identified in *S. constricta*. This was in contrast to pre-existing knowledge that there was a single Elovl4 isoform characterized from *C. nobilis* and *O. vulgaris* [21, 22], but was consistent with findings in most teleosts [6, 39]. More importantly, a novel *S. constricta* Elovl (Elovl_c) with Elovl4 activity, sharing similar phylogeny with some marine molluscan Elovl-like (not functionally characterized), was grouped closely to vertebrate Elovl1 and Elovl7, indicating that they couldn't be defined as Elovl4 strictly and might be recognized as novel Elovl members.

The phylogenic results of Elovl suggested that Elovl2/5 and Elovl4 might all be present in the six examined marine molluscs. To further understand their capacity to biosynthesize LC-PUFA, a phylogenetic tree regarding Fad was also constructed (Fig. S3). The resulting tree indicated that Δ5 Fad might also be a common gene in the six examined marine molluscs, but this was not the case with Δ4, Δ6 and Δ8 Fad. Notably, Δ4 Fad could directly desaturate 22:5n-3 to produce DHA compared with the more complicated Sprecher pathway [40]. Therefore, a similar conclusion with that of Monroig et al [22] can be reached that the limited DHA biosynthetic capability of marine molluscs might be a consequence of the lack of Δ4 or Δ6 Fad. However, multiple functional genes might also exist in marine molluscs to complete the Δ4 or Δ6 Fad desaturation step, as in some teleosts [6], but this requires further investigation. Importantly, no multiple functional Fad genes were found in *S. constricta* [28].

Furthermore, multiple transcripts of Elovl4 and Δ5 Fad might be a common phenomenon in some marine molluscs, indicating that lineage specific duplication events might have occurred in the evolution and expansion of those genes in these species but not in the cephalopods.

Similar to Elovl2/5 from *O. vulgaris*, *S. officinalis*, *C. nobilis* and *C. angulate* [17-20], the elongation efficiencies of *S. constricta* Elovl2/5 toward n-6 LC-PUFA were higher than toward the homologous n-3 LC-PUFA. This indicated that n-6 LC-PUFA might be particularly important for the normal development of marine molluscs including *S. constricta*. From another point of view, it might be associated with their living conditions that the easy availability of n-3 LC-PUFA of natural diets might influence the corresponding activities of Elovl2/5. Previously, it was hypothesized that the elongation activity of Elovl4 toward C_{22} might be an adaptive strategy to compensate the loss of Elovl2 activity in several molluscan Elovl2/5 [12], and it may be similar with *S. constricta*.

The retention of three transcripts with Elovl4 activity in *S. constricta* might have resulted from their respective substrate specificities and specific tissues distributions. The elongated PUFA of different carbon chain length could have critical physiological significance for this bivalve, which requires further investigations. Importantly, the three *S. constricta* Elovl all possessed the ability to elongate 20:5n-3 and 22:5n-3 to give 24:5n-3, which can be further desaturated to 24:6n-3 by *S. constricta* Δ6 Fad. Although DHA production from *β*-oxidation of 24:6n-3 was not determined directly, it is speculated that DHA could be biosynthesized in this bivalve. To confirm this

hypothesis, further research using radiolabeled C_{18} precursor or feeding diets lacking DHA is required.

Consistent with the tissue distributions of *S. constricta* Fad [37], intestine and gonad exhibited relatively high expressions of *S. constricta* Elovl, indicating that the two tissues might be major metabolic sites of LC-PUFA in this bivalve species. Interestingly, the highest expressions of *S. constricta* Elovl2/5, Elovl4_a and Elovl_c were found in digestive glands, gill and mantle, respectively. In contrast, the highest expression of *S. constricta* Elovl4_b was detected in both labial palps and gill. The results suggested that the digestive glands might be an auxiliary organ of LC-PUFA biosynthesis, and LC-PUFA (especially $\geq C_{24}$) might be necessary for mantle, labial palps and gill to execute their respective physiological roles.

In summary, *S. constricta* was demonstrated as the first marine mollusc with all Fad and Elovl activities required for LC-PUFA biosynthesis via the Sprecher pathway, which greatly increases our understanding of LC-PUFA biosynthesis in marine molluscs. Based on this finding, it was speculated that DHA could be endogenously biosynthesized in this important bivalve species. Meanwhile, the phylogenic results suggested that the DHA biosynthetic ability might be limited to a few specific molluscan species, with inability in many species mainly attributed to the lack of Δ4 or Δ6 Fad.

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Figures

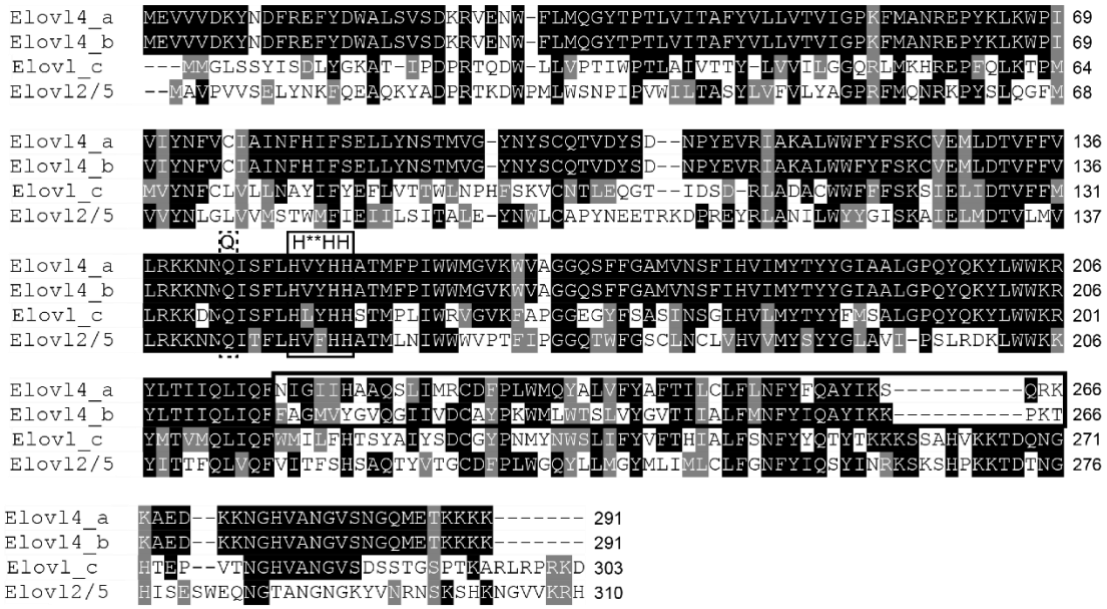

Fig. 1. Amino acid (aa) sequence alignment of *S. constricta* Elovl2/5, Elovl4_a, Elovl4_b and Elovl_c by using ClustalW 2.1. The histidine box (H**HH) conserved among Elovl family members is highlighted with a solid line square. The diagnostic "Q" conserved in PUFA elongase is highlighted with a dotted line square. The different aa domains between *S. constricta* Elovl4_a and Elovl4_b are indicated with a bold line square.

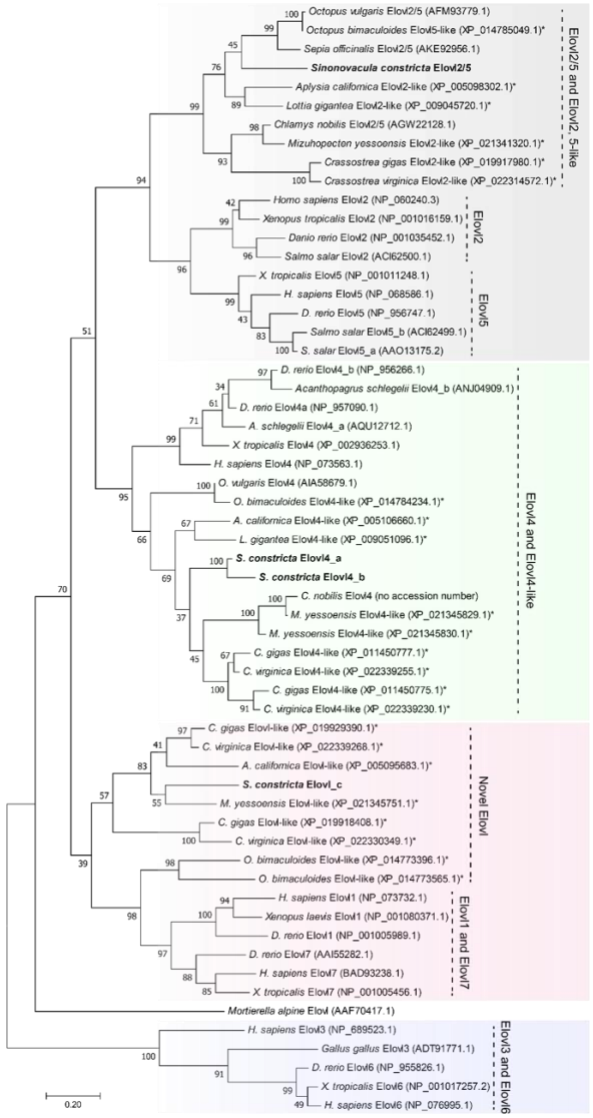

Fig. 2. Phylogenetic tree comparing the deduced aa sequences of *S. constricta* Elovl (bold fonts) with Elovl1-7 proteins from representative mammals, amphibians, fish and marine molluscs. The tree was constructed using the maximum-likelihood approach with MEGA 7. The horizontal branch length is proportional to aa substitution rate per site. The numbers represent the frequencies with which the tree topology presented was replicated after 1,000 iterations. An asterisk indicates Elovl genes of *A. californica*, *C. gigas*, *C. virginica*, *L. gigantea*, *M. yessoensis* and *O. bimaculoides* that have not been functionally characterized but their complete genomes are currently available (Table S2).

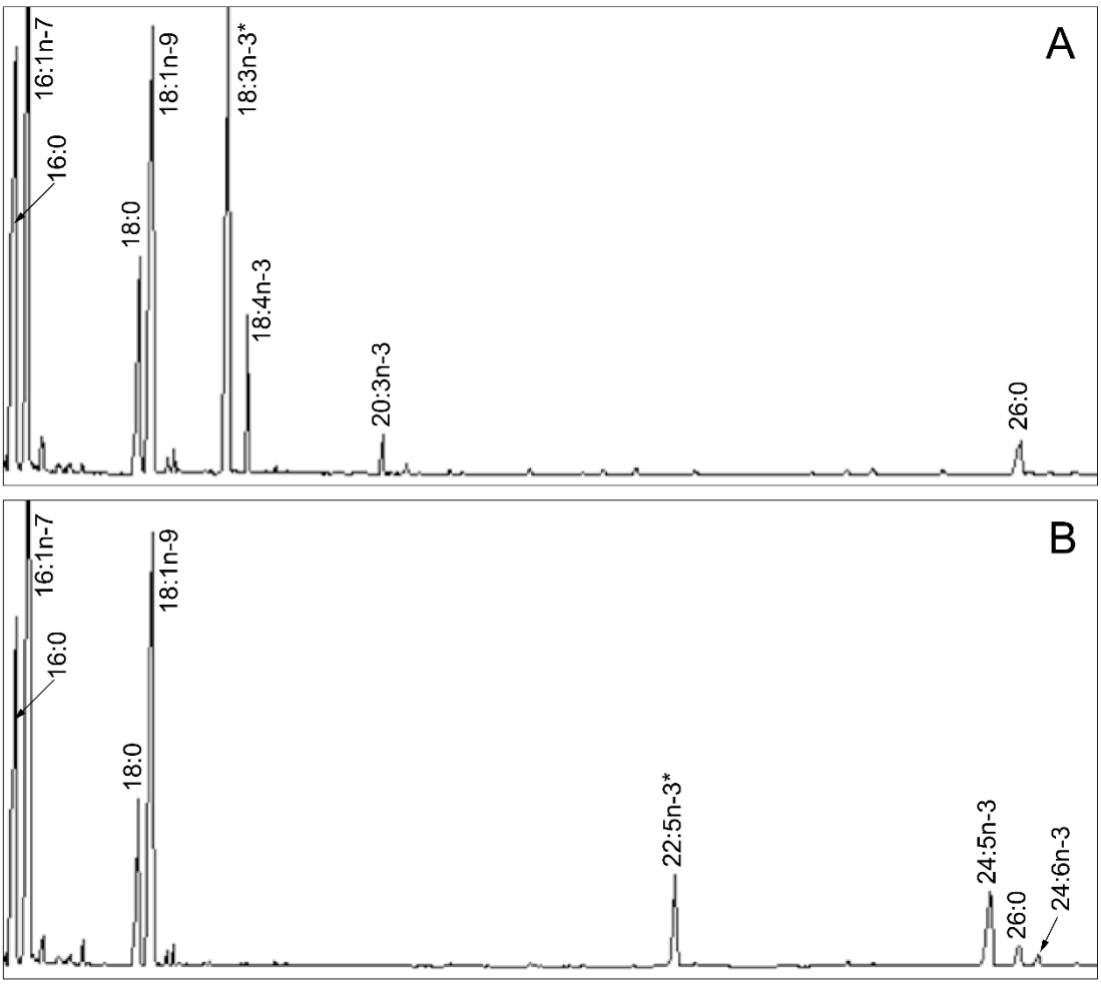

Fig. 3. Characterization of the ability of *S. constricta* Δ6 Fad for Δ6 desaturation towards 24:5n-3 using yeast *Saccharomyces cerevisiae*. Fatty acid (FA) profiles of yeast co-transformed with the ORF of *S. constricta* Δ6 Fad and that of the zebrafish *Danio rerio* Elovl2 and grown in the presence of an exogenously added FA substrates (indicated as "*" in panels A and B). The 16:0, 16:1n-7, 18:0, 18:1n-9 and 26:0 are major yeast endogenous FA. Elongation (20:3n-3 and 24:5n-3) and Δ6 desaturation (18:4n-3 and 24:6n-3) products from exogenously added or endogenously produced FA are indicated accordingly.

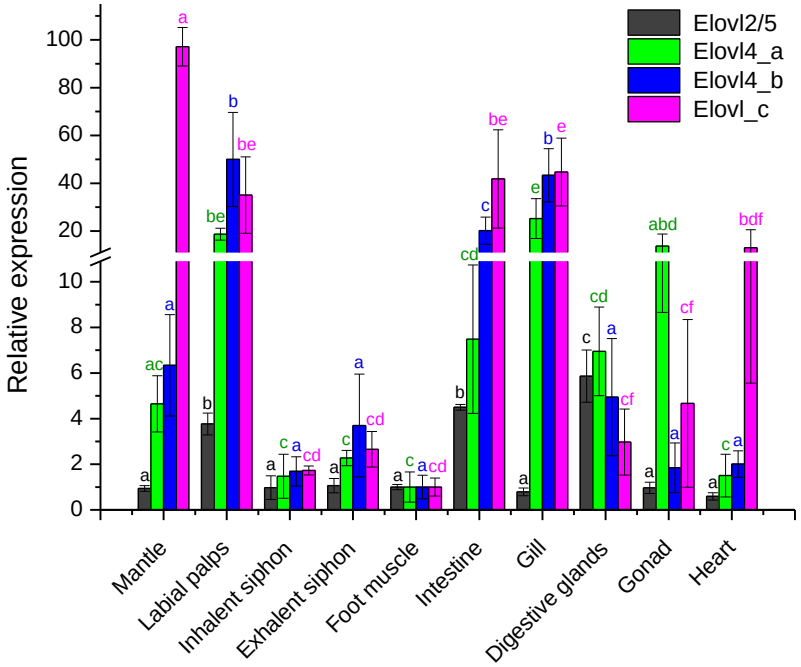

Fig. 4. Tissue distributions of *S. constricta* Elovl. Expressions of *S. constricta* Elovl were examined by qPCR and normalized by *β-actin*. Relative expression of *S. constricta* Elovl in tissues were in relation to the normalized expression data in foot muscle, respectively. The values (means ± SD, n=3) sharing a common letter within the same color were not significantly different ($P \leq 0.05$).

Tables

Table 1 Primers used for *S. constricta* Elovl partial sequence verification (1st fragment PCR), full length cloning (RACE PCR), plasmid construction (ORF cloning) and tissue distribution (qPCR), respectively. The restriction sites of *Kpn*I and *Eco*RI are underlined.

transcript	aim	primer	sequence
Elovl2/5	1st fragment PCR	V-F-Elovl2/5	ATGGCTGTTCCTGTTGTGTCAGA
		V-R-Elovl2/5	CACCACAGAAATCAGATGATTTTACA
	RACE PCR	5'RACE-outer	CCATGTTGACATGACCACCAGTCCGAG
		5'RACE-inner	CGTCGATAACCACCCAGCGATCGTC
		3'RACE-outer	TACTGTCTAGGGATTTAATTGGGCAGCC
		3'RACE-inner	TGTAAAATCATCTGATTTCTGTGGTG
	ORF cloning	Elovl2/5-F	GGGGTACCATGGCTGTTCCTGTTGTGTCAGA
		Elovl2/5-R	CGGAATTCCTAATGTCTTTTGACTACACCATTTTTATGAC
Elovl4_a/b	1st fragment PCR	V-F-Elovl4_a/b	GAGTTCTATGACTGGGCCCTCT
		V-R-Elovl4_a/b	CCATCTGTCCATTGGACACTCC
	RACE PCR	5'RACE-outer	GCCATTTGAGCTTGTATGGTTCCCGAT
		5'RACE-inner	GTTCTCCACACGCTTGTCTGCGACG
		3'RACE-outer	GAAGCGCTACCTCACCATTATCCAACTG
		3'RACE-inner	GCATGTGGCAAATGGAGTGTCCAATG
	ORF cloning	Elovl4_a/b-F	GGGGTACCATGGAGGTGGTAGTAGACAAGTACA
		Elovl4_a/b-R	CGGAATTCTTACTTCTTTTTCTTGGTTTCCATC
Elovl_c	1st fragment PCR	V-F-Elovl_c	GGGACTGTCTAGCTACATCAGTGATC
		V-R-Elovl_c	CTGTTGATGAATCACTGACGCC
	RACE PCR	5'RACE-outer	GTCCTCCAAGGATGACCACAAGGTAAGTG
		5'RACE-inner	AAGCAACCAGTCCTGGGTACGTGGAT

	3'RACE-outer	GCTCTGCACACGTCAAGAAAACTGACCA
	3'RACE-inner	GACATGTTGCCAATGGCGTCAGTGAT
ORF cloning	Elovl_c-F	GGGGTACCATGATGGGACTGTCTAGCTACATCA
	Elovl_c-R	CGGAATTCCTAATCCTTTCTTGGACGTAATCGT
qPCR	Elovl2/5-qF	GCTCAACATTTGGTGGTGGGT
	Elovl2/5-qR	GGAATGACTGCCAGACCGTAG
	Elovl4_a-qF	TTGGGATCATTCACGCAGCC
	Elovl4_a-qR	GATGGTGAATGCGTAAAACACAAGA
	Elovl4_b-qF	TGCCGGTATGGTCTACGGTGT
	Elovl4_b-qR	GATTGTGACACCGTATACAAGCGAG
	Elovl_c-qF	TGCTATCTACTCGGACTGTGGC
	Elovl_c-qR	GTTTTCTTGACGTGTGCAGAGC
	β-actin-qF	CCATCTACGAAGGTTACGCCC
	β-actin-qR	TCGTAGTGAAGGAGTAGCCTCTTTC
	18S-rRNA-qF	ATGCTTTCGCTGTAGTTCGTCTTG
	18S-rRNA-qR	CTCGGTTCTATTGCGTTGGTTTT

Table 2 Functional characterization of *S. constricta* Elovl2/5: conversions of PUFA substrates. Individual conversion rates were calculated according to the formula [areas of first product and longer chain products / (areas of all products with longer chain than substrate + substrate area)] × 100%.

PUFA substrate	Product	% Conversion	Activity
18:2n-6	20:2n-6	30.03	$C_{18\rightarrow22}$
	22:2n-6	9.49	$C_{20\rightarrow22}$
18:3n-3	20:3n-3	20.65	$C_{18\rightarrow22}$
	22:3n-3	3.50	$C_{20\rightarrow22}$
18:3n-6	20:3n-6	28.56	$C_{18\rightarrow22}$
	22:3n-6	11.66	$C_{20\rightarrow22}$
18:4n-3	20:4n-3	11.73	$C_{18\rightarrow22}$
	22:4n-3	5.24	$C_{20\rightarrow22}$
20:4n-6	22:4n-6	39.54	$C_{20\rightarrow22}$
20:5n-3	22:5n-3	14.98	$C_{20\rightarrow22}$
22:4n-6	24:4n-6	n.d.	$C_{22\rightarrow24}$
22:5n-3	24:5n-3	n.d.	$C_{22\rightarrow24}$

n.d., not detected.

Table 3 Functional characterization of *S. constricta* Elovl4_a, Elovl4_b and Elovl_c: conversions of PUFA substrates. Individual conversion rates were calculated according to the formula [areas of first product and longer chain products / (areas of all products with longer chain than substrate + substrate area)] × 100%.

PUFA substrate	Product	% Conversion			Activity
		pY*elovl4_a*	pY*elovl4_b*	pY*elovl_c*	
18:2n-6	20:2n-6	0.72	0.37	0.62	$C_{18\rightarrow20}$
18:3n-3	20:3n-3	4.22	1.57	3.66	$C_{18\rightarrow20}$
18:3n-6	20:3n-6	0.57	0.47	4.37	$C_{18\rightarrow22}$
	22:3n-6	n.d.	n.d.	3.00	$C_{20\rightarrow22}$
18:4n-3	20:4n-3	0.90	0.49	5.25	$C_{18\rightarrow22}$
	22:4n-3	n.d.	n.d.	2.32	$C_{20\rightarrow22}$
20:4n-6	22:4n-6	1.29	1.20	1.91	$C_{20\rightarrow26}$
	24:4n-6	n.d.	10.43	37.21	$C_{22\rightarrow26}$
	26:4n-6	n.d.	n.d.	61.81	$C_{24\rightarrow26}$
20:5n-3	22:5n-3	1.65	100	6.15	$C_{20\rightarrow34}$
	24:5n-3	21.8	0.44	78.54	$C_{22\rightarrow34}$
	26:5n-3	100	26.89	85.75	$C_{24\rightarrow34}$
	28:5n-3	100	n.d.	31.36	$C_{26\rightarrow34}$
	30:5n-3	100	n.d.	7.31	$C_{28\rightarrow34}$
	32:5n-3	55.39	n.d.	100	$C_{30\rightarrow34}$
	34:5n-3	27.06	n.d.	28.73	$C_{32\rightarrow34}$
22:4n-6	24:4n-6	3.12	1.18	2.22	$C_{22\rightarrow30}$
	26:4n-6	37.11	n.d.	32.33	$C_{24\rightarrow30}$
	28:4n-6	100	n.d.	n.d.	$C_{26\rightarrow30}$
	30:4n-6	42.16	n.d.	n.d.	$C_{28\rightarrow30}$
22:5n-3	24:5n-3	10.14	2.13	14.94	$C_{22\rightarrow34}$
	26:5n-3	59.89	n.d.	68.01	$C_{24\rightarrow34}$

	28:5n-3	100	n.d.	18.35	$C_{26 \rightarrow 34}$
	30:5n-3	68.45	n.d.	n.d.	$C_{28 \rightarrow 34}$
	32:5n-3	68.51	n.d.	n.d.	$C_{30 \rightarrow 34}$
	34:5n-3	16.42	n.d.	n.d.	$C_{32 \rightarrow 34}$

n.d., not detected.

Supplementary Tables

Table S1 Summary of conditions of PCR.

Aim	Polymerase	Conditions
First fragment PCR	TaKaRa LA Taq HS	an initial denaturation step at 94℃ for 30 s, followed by 30 cycles of denaturation at 98℃ for 10 s, annealing and extension at 68℃ for 1 min, followed by a final extension at 72℃ for 10 min.
RACE PCR	SeqAmp DNA Polymerase	first-round (touch-down PCR): 5 cycles of 94℃ for 30 s and 72℃ for 3 min, 5 cycles of 94℃ for 30 s, 70℃ for 30 s and 72℃ for 3 min, 25 cycles of 94℃ for 30s, 68℃ for 30 s and 72℃ for 3min.
	SeqAmp DNA Polymerase	second-round (touch-down PCR): 5 cycles of 94℃ for 30 s and 72℃ for 3 min, 5 cycles of 94℃ for 30 s, 70℃ for 30 s and 72℃ for 3 min, 25 cycles of 94℃ for 30s, 68℃ for 30 s and 72℃ for 3min.
ORF cloning	PrimeSTAR GXL	30 cycles of 98℃ for 10 s, 55℃ for 15 s and 68℃ for 1 min.
qPCR	SYBR *Premix Ex Taq*	an initial denaturation step at 95℃ for 30 s, followed by 35 cycles of 95℃ for 5 s, 55℃ for 15 s and 72℃ for 20 s, followed by a melting curve from 58°C to 95°C with an increment of 1.85°C/min.

Table S2 Protein sequences from potential Elovl and Fad involved in LC-PUFA biosynthesis in six marine molluscs, namely *A. californica* (NCBI, genome ID: 443), *C. gigas* (NCBI, genome ID: 10758), *C. virginica* (NCBI, genome ID: 398), *L. gigantea* (NCBI, genome ID: 15113), *M. yessoensis* (NCBI, genome ID: 12193) and *O. bimaculoides* (NCBI, genome ID: 41501).

Species	Gene	Gene ID	Prediction in database
A. californica (genome ID: 443)	Elovl	XP_005098302.1	elongation of very long chain fatty acids protein 2-like isoform X3
		XP_005106660.1	elongation of very long chain fatty acids protein 4-like2
		XP_005095683.1	elongation of very long chain fatty acids protein 4-like
	Fad	XP_005090573.1	fatty acid desaturase 1-like isoform X1
		XP_005090577.1	fatty acid desaturase 1-like isoform X2
		XP_012941605.1	fatty acid desaturase 2-like
		XP_005097048.1	fatty acid desaturase 2-like
		XP_005093182.1	fatty acid desaturase 2-like
C. gigas (genome ID: 10758)	Elovl	XP_019917980.1	elongation of very long chain fatty acids protein 2-like
		XP_011450777.1	elongation of very long chain fatty acids protein 4 isoform X2
		XP_011450775.1	elongation of very long chain fatty acids protein 4 isoform X1
		XP_019929390.1	elongation of very long chain fatty acids protein 4
		XP_019918408.1	elongation of very long chain fatty acids protein 4
	Fad	XP_011414051.1	fatty acid desaturase 2 isoform X2
		XP_011414050.1	fatty acid desaturase 2 isoform X1
		XP_011430931.1	fatty acid desaturase 2
C. virginica (genome ID: 398)	Elovl	XP_022314572.1	elongation of very long chain fatty acids protein 2-like
		XP_022339255.1	elongation of very long chain fatty acids protein 4-like isoform X2

		XP_022339230.1	elongation of very long chain fatty acids protein 4-like isoform X1
		XP_022339268.1	elongation of very long chain fatty acids protein 4-like
		XP_022330349.1	elongation of very long chain fatty acids protein 4-like isoform X2
	Fad	XP_022323911.1	fatty acid desaturase 2-like isoform X3
		XP_022323910.1	fatty acid desaturase 2-like isoform X2
		XP_022320667.1	fatty acid desaturase 2-like
L. gigantea	Elovl	XP_009045720.1	elongation of very long chain fatty acids protein 5
(genome ID:	Fad	XP_009051096.1	elongation of very long chain fatty acids protein 4
15113)		XP_009049968	fatty acid desaturase like
		XP_009045077	fatty acid desaturase like
		XP_009051231	fatty acid desaturase 2 like
M. yessoensis	Elovl	XP_021341320.1	elongation of very long chain fatty acids protein 2-like
(genome ID:		XP_021345829.1	elongation of very long chain fatty acids protein 4-like isoform X1
12193)		XP_021345830.1	elongation of very long chain fatty acids protein 4-like isoform X2
		XP_021345751.1	elongation of very long chain fatty acids protein 4-like
	Fad	XP_021370917.1	fatty acid desaturase 2-like
		XP_021351912.1	fatty acid desaturase 2-like
O. bimaculoides	Elovl	XP_014785049.1	elongation of very long chain fatty acids protein 5-like isoform X2
(genome ID:			
41501)		XP_014784234.1	elongation of very long chain fatty acids protein 4-like isoform X2
		XP_014773396.1	elongation of very long chain fatty acids protein 4-like
		XP_014773565.1	elongation of very long chain fatty acids protein 4-like
	Fad	XP_014768102.1	fatty acid desaturase 2-like

Table S3 Functionally characterized Elovl and Fad cDNAs from marine molluscs including *C. nobilis*, *H. discus hannai*, *O. vulgaris*, *S. officinalis*, *S. constricta* and *C. angulata.*

Species	Gene	Description	Accession number
C. nobilis	Elovl	Elovl2/5	AGW22128.1
		Elovl4	no accession
	Fad	Δ5 Fad	AIC34709
		Δ8 Fad	no accession
H. discus hannai	Fad	Δ5 Fad_a	ADK38580
		Δ5 Fad_b	ADK12703
O. vulgaris	Elovl	Elovl2/5	AFM93779.1
		Elovl4	AIA58679.1
	Fad	Δ5 Fad	AEK20864
S. officinalis	Elovl	Elovl2/5	AKE92956.1
	Fad	Δ5 Fad	AKE92955
S. constricta	Fad	Δ5 Fad_a	MH220404
		Δ5 Fad_b	MH220405
		Δ6 Fad	MH220406
C. angulata	Elovl	Elovl2/5	no accession

Supplementary Figures

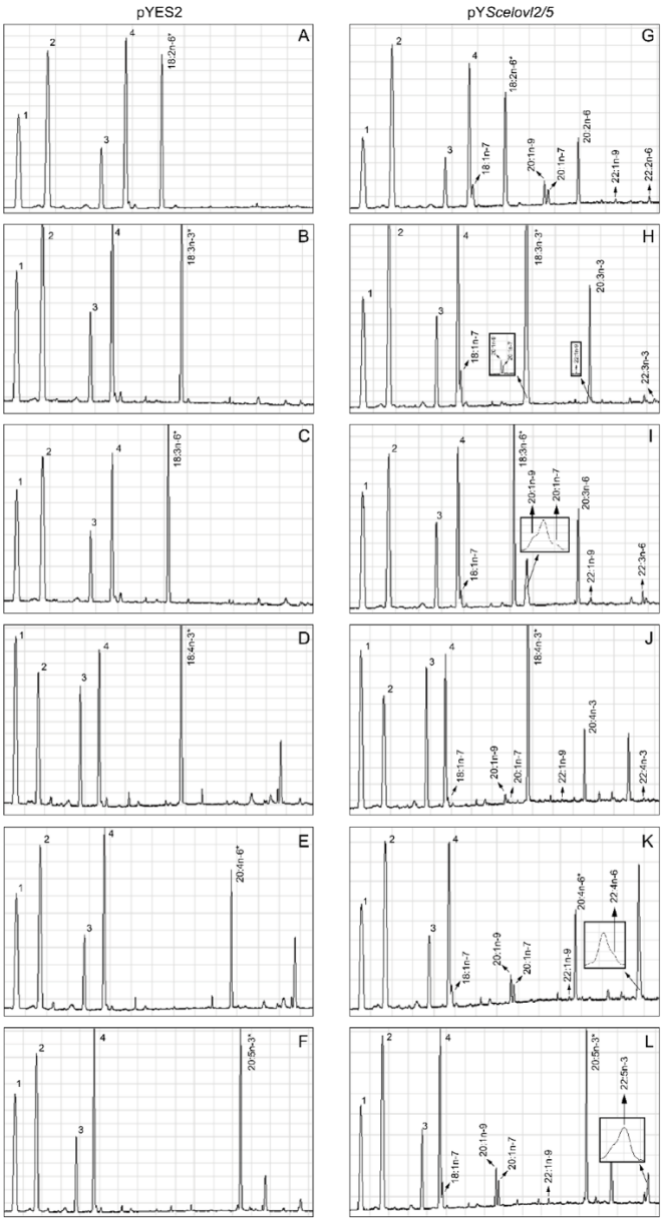

Fig. S1. Functional characterization of *S. constricta* Elovl2/5. Fatty acid methyl esters were prepared from total lipids of yeast transformed with pYES2 alone (A-F) or pYES2 expressing the *S. constricta* Elovl2/5 (*pYScelovl2/5*) (G-L). Peaks 1-4 are the main endogenous fatty acids (FAs) of *S. cerevisiae*, namely, 16:0, 16:1n-7, 18:0, and 18:1n-9, respectively. The exogenously added FAs are highlighted with *. The FA products are also indicated in the corresponding panels. Note that 18:1n-7 was the elongation product of the yeast endogenous 16:1n-7. Similarly, 20:1n-9 and 22:1n-9 are the elongation products of the yeast endogenous 18:1n-9.

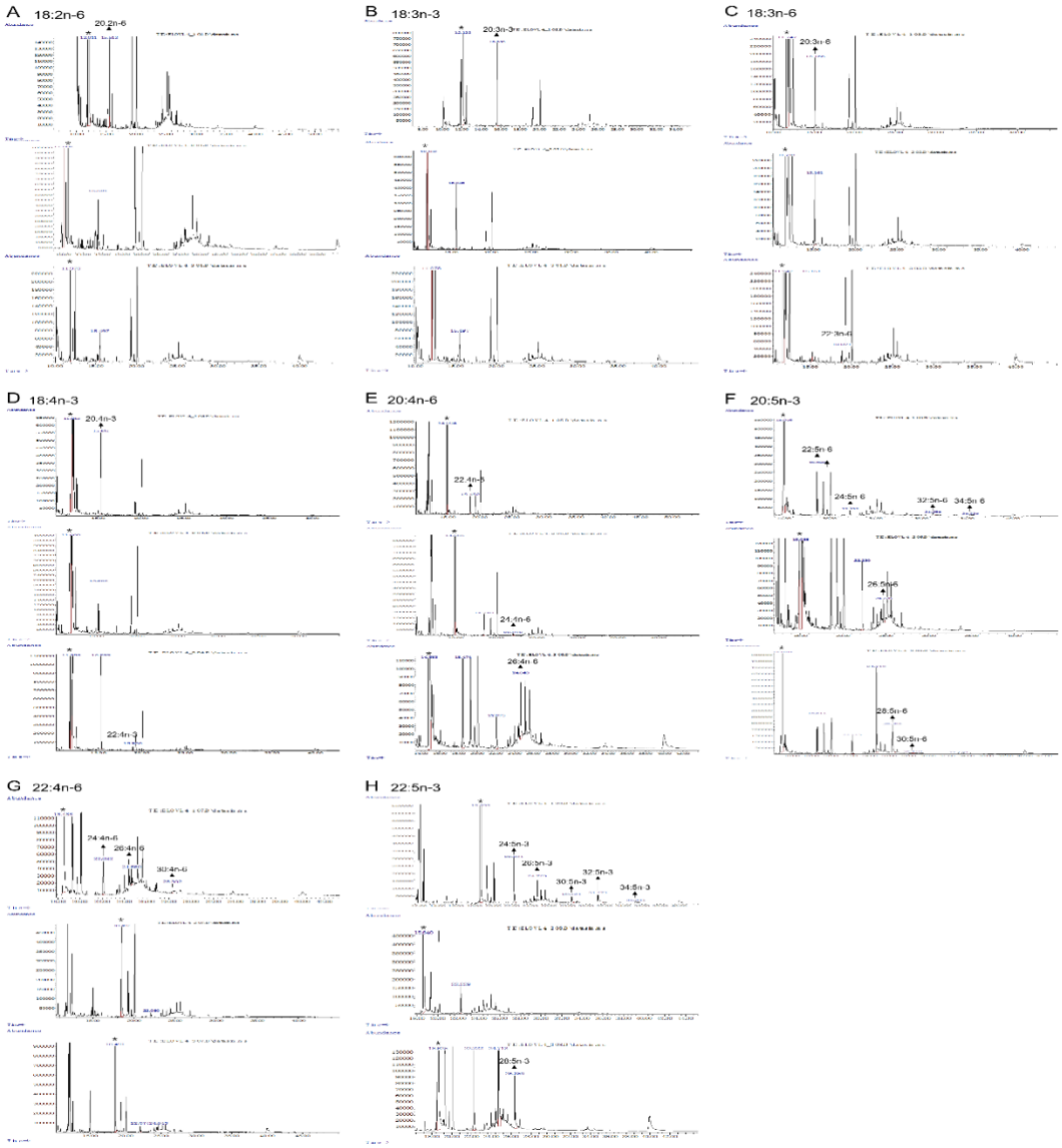

Fig. S2. Functional characterization of *S. constricta* Elovl4_a, Elovl4_b and Elovl_c. The exogenously added fatty acid (FA) substrates are highlighted with * and indicated following the capital letters. Each set of three chromatograms correspond to results obtained by expressing the *S. constricta* Elovl4_a (top) Elovl4_b (middle) and Elovl_c (bottom). All elongation products are indicated in each panel.

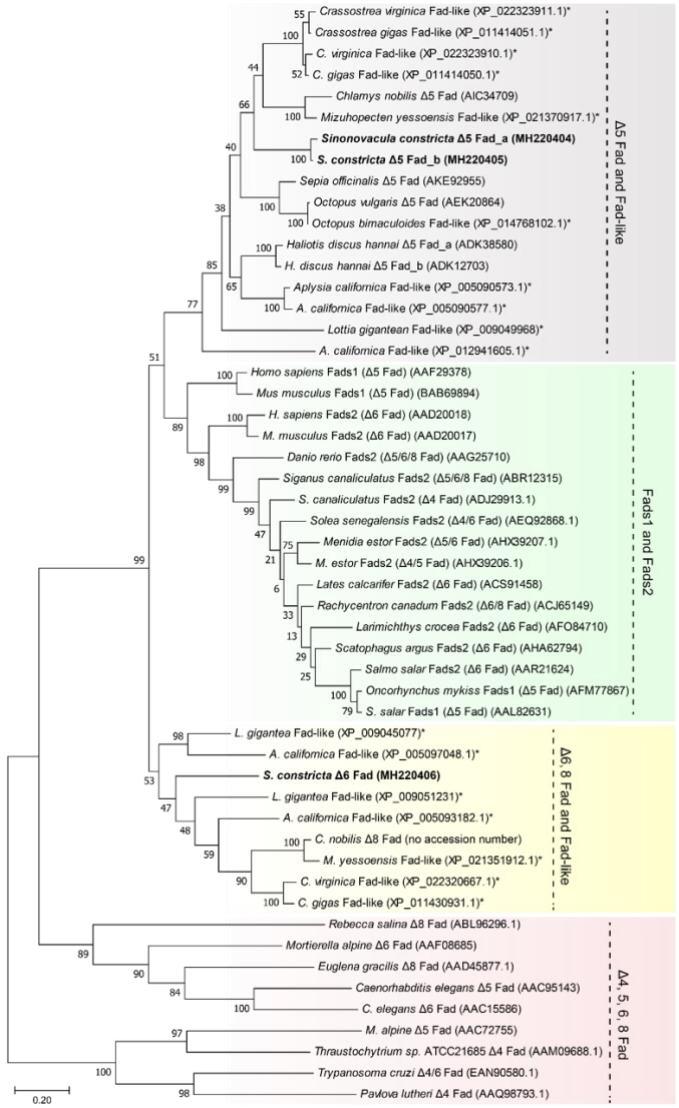

Fig. S3. Phylogenetic tree comparing the deduced aa sequences of *S. constricta* Δ5 Fad_a, Δ5 Fad_b and Δ6 Fad (bold fonts) with Δ4, 5, 6, 8 Fad proteins from representative mammals, fish, marine molluscs and other lower eukaryotes. The tree was constructed using the maximum-likelihood approach with MEGA 7. The horizontal branch length is proportional to aa substitution rate per site. The numbers represent the frequencies with which the tree topology presented was replicated after 1,000 iterations. An asterisk indicates the Fad genes of *A. californica*, *C. gigas*, *C. virginica*, *L. gigantea*, *M. yessoensis* and *O. bimaculoides* that have not been functionally characterized but their complete genomes are currently available (Table S2).